New data letter

DfrA-thyA double deletion in *para*-aminosalicylic acid resistant *Mycobacterium tuberculosis* Beijing strains

Running title: PAS resistance due to *dfrA-thyA* deletion

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Para-aminosalicylic acid (PAS) is a group 4 anti-tuberculosis agent (1). It targets folate metabolism as shown in Fig. S1, which also summarises the known resistance mechanisms to this pro-drug (2). Recently, we reported a multidrug-resistant (MDR) *Mycobacterium tuberculosis* Beijing strain harbouring a deletion of both *dfrA* and *thyA* from Australia (Fig. 1A and Table S1) (3). Since then, we have found deletions affecting both genes in five further MDR Beijing strains (two isolated in Australia and three from Peru) and one extensively drug-resistant (XDR) Beijing strain from China. The Australian MDR strains were recovered from three patients with no apparent epidemiological links and were likely infected in their country of origin (Table S1). The three Peruvian isolates were closely related and consequently shared the same deletion, whereas the remaining strains were distantly related and had deletions that differed in size (Fig. 1A). Consequently, these five distinct deletions were acquired independently, which can be a signal for positive selection of resistance mechanisms. In line with this hypothesis, the strains from Australia and China were PAS resistant when tested with the BACTEC MGIT 960 system and on Löwenstein-Jensen medium, respectively (Supplementary Methods). Two out of the three Peruvian deletion mutants were also PAS resistant on 7H10 medium at 8 µg/mL, whereas the two closely related ancestral wild-type strains were susceptible (Fig. 1B). We were unable to retest the strains at 2 µg/mL, the recommended critical concentration by the Clinical and Laboratory Standards Institute and World Health Organization, which would have clarified whether the susceptible result for the third deletion mutant was an artefact (1, 4).

The observation that *dfrA* could be deleted was remarkable in light of our current understanding of folate metabolism in *M. tuberculosis*. Two studies suggested that *dfrA* is essential *in vitro* in the H37Rv laboratory strain (5, 6). More recently, it was shown that *dfrA* is conditionally essential and can only be knocked out in H37Rv if Rv2671 is over-expressed *in trans*, presumably due to its much lower dihydrofolate reductase activity compared with DfrA (7, 8). Our *in silico* analysis of the seven *dfrA-thyA* double deletion mutants did not reveal any known *Rv2671* mutations (Table S1), such as the G to A upstream mutation at position -12 that results in its over-expression and consequently confers PAS resistance (this mutation was incorrectly referred to as affecting position -11 in two of our prior studies (7, 9)). Assuming that no other pertinent differences exist that are specific to the Beijing genotype relative to H37Rv or that a yet unknown acquired mutation elsewhere in the genome were present that resulted in the over-expression of *Rv2671*, we propose that this apparent contradiction can be reconciled if the essentiality of *dfrA* was not only dependent on the expression level of *Rv2671*, but also on the presence of wild-type *thyA*. The fact that *thyA* was deleted in all seven *dfrA* mutants meant that only the second thymidylate synthase, encoded by the essential *thyX*, was active in these strains (Fig. S1). Contrary to ThyA, ThyX generates tetrahydrofolate rather than dihydrofolate upon catalysis and therefore does not require high dihydrofolate reductase activity to provide sufficient levels of tetrahydrofolate (2). This is in line with the fact that *dfrA* is not required in bacterial species that lack *thyA* (10). Consequently, *Rv2671* appeared to be sufficient to sustain growth, even without being over-expressed in these deletion mutants. It should therefore be possible to knock out *dfrA* in strains of *M. tuberculosis* with inactive *thyA*. Moreover, the adjacent location of *thyA* and *dfrA* in genome should make their simultaneous deletion possible (Fig. 1A).

Interestingly, all but one of the deletion mutants also convergently acquired mutations upstream of *thyX* compared to the two closely related Peruvian control strains (Fig. 1B & Table S1) (11). In fact, the cluster of three Peruvian strains and two of the unrelated Australian strains shared the same C to T upstream mutation at position -16 that has been previously found to be associated with resistance to several drugs and experimentally shown to result in the over-expression of *thyX* (12). It is therefore plausible that these changes compensated for the reduced expression levels and enzymatic activity of ThyX compared to ThyA (11, 13). Based on our data, however, it was not

possible to deduce whether the *thyX* mutations were acquired after the deletions of *thyA* and *dfrA* in each strain, as would be expected with compensatory mutations (11).

In summary, these data demonstrated that the folate metabolism and the genetic basis of PAS resistance are more complex than previously appreciated, which is relevant for the development of novel DfrA and ThyX inibitors and potentially the use of trimethoprim-sulfamethoxazole to treat drug-resistant tuberculosis (Fig. S1) (14-25). Although deletions are often excluded from large-scale whole genome studies owing to the limited read lengths next-generation sequencers and the fact that algorithms are optimised for SNP calling, this study highlighted that deletions can no longer be ignored (3, 26).

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Fig. 1. Analysis of *dfrA* and *thyA* deletion strains, all of which tested PAS resistant, with the exception of PH107_CA033M_1. (a) Diagram of deletions in seven clinical strains compared with the wild-type H37Rv laboratory strain. The scale at the top corresponds to the genome position in H37Rv. The letter in parentheses denotes the country of isolation (Australia (A), China (C), and Peru (P)). Mtb97 was reported previously (3). (b) Maximum likelihood tree based on whole-genome data of the three Peruvian deletion mutants, which also share a mutation upstream of *thyX* that is also present in Mtb97 and Mtb78 (Table S1), and two closely related wild-type strains, which were PAS susceptible.

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Conflicts of interest

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References

1. **World Health Organization.** Companion handbook to the WHO guidelines for the programmatic management of drug-resistant tuberculosis (2014).

http://apps.who.int/iris/bitstream/10665/130918/1/9789241548809_eng.pdf?ua=1&ua=1 (accessed 13.8.2015).

2. **Minato Y, Thiede JM, Kordus SL, McKlveen EJ, Turman BJ, Baughn AD.** 2015. *Mycobacterium tuberculosis* folate metabolism and the mechanistic basis for *para*-aminosalicylic acid susceptibility and resistance. Antimicrob Agents Chemother **59:**5097-5106.

3. **Martinez E, Holmes N, Jelfs P, Sintchenko V.** 2015. Genome sequencing reveals novel deletions associated with secondary resistance to pyrazinamide in MDR *Mycobacterium tuberculosis*. J Antimicrob Chemother **70:**2511-2514.

4. **Clinical and Laboratory Standards Institute.** 2011. Susceptibility testing of mycobacteria, nocardiae, and other aerobic actinomycetes, 2nd edition Approved standard. CLSI document M24-A2.

5. **Griffin JE, Gawronski JD, Dejesus MA, Ioerger TR, Akerley BJ, Sassetti CM.** 2011. High-resolution phenotypic profiling defines genes essential for mycobacterial growth and cholesterol catabolism. PLoS Pathog **7:**e1002251.

6. **Sassetti CM, Boyd DH, Rubin EJ.** 2003. Genes required for mycobacterial growth defined by high density mutagenesis. Mol Microbiol **48:**77-84.

7. **Zheng J, Rubin EJ, Bifani P, Mathys V, Lim V, Au M, Jang J, Nam J, Dick T, Walker JR, Pethe K, Camacho LR.** 2013. *Para*-aminosalicylic acid is a prodrug targeting dihydrofolate reductase in *Mycobacterium tuberculosis*. J Biol Chem **288:**23447-23456.

8. **Cheng YS, Sacchettini JC.** 2016. Structural insights into *Mycobacterium tuberculosis* Rv2671 protein as a dihydrofolate reductase functional analogue contributing to *para*-aminosalicylic acid resistance. Biochemistry **55:**1107-1119.

9. **Zhang X, Liu L, Zhang Y, Dai G, Huang H, Jin Q.** 2015. Genetic determinants involved in *p*-aminosalicylic acid resistance in clinical isolates from tuberculosis patients in northern China from 2006 to 2012. Antimicrob Agents Chemother **59:**1320-1324.

10. **Myllykallio H, Leduc D, Filee J, Liebl U.** 2003. Life without dihydrofolate reductase FolA. Trends Microbiol **11:**220-223.

11. **Merker M, Kohl TA, Roetzer A, Truebe L, Richter E, Rüsch-Gerdes S, Fattorini L, Oggioni MR, Cox H, Varaine F, Niemann S.** 2013. Whole genome sequencing reveals complex evolution patterns of multidrug-resistant *Mycobacterium tuberculosis* Beijing strains in patients. PLoS One **8:**e82551.

12. **Zhang H, Li D, Zhao L, Fleming J, Lin N, Wang T, Liu Z, Li C, Galwey N, Deng J, Zhou Y, Zhu Y, Gao Y, Wang T, Wang S, Huang Y, Wang M, Zhong Q, Zhou L, Chen T, Zhou J, Yang R, Zhu G, Hang H, Zhang J, Li F, Wan K, Wang J, Zhang XE, Bi L.** 2013. Genome sequencing of 161 *Mycobacterium tuberculosis* isolates from China identifies genes and intergenic regions associated with drug resistance. Nat Genet **45:**1255-1260.

13. **Fivian-Hughes AS, Houghton J, Davis EO.** 2012. *Mycobacterium tuberculosis* thymidylate synthase gene *thyX* is essential and potentially bifunctional, while *thyA* deletion confers resistance to *p*-aminosalicylic acid. Microbiology **158:**308-318.

14. **Kögler M, Busson R, De Jonghe S, Rozenski J, Van Belle K, Louat T, Munier-Lehmann H, Herdewijn P.** 2012. Synthesis and evaluation of 6-aza-2'-deoxyuridine monophosphate analogs as inhibitors of thymidylate synthases, and as substrates or inhibitors of thymidine monophosphate kinase in *Mycobacterium tuberculosis*. Chem Biodivers **9:**536-556.

15. **Kumar A, Zhang M, Zhu L, Liao RP, Mutai C, Hafsat S, Sherman DR, Wang MW.** 2012. High-throughput screening and sensitized bacteria identify an *M. tuberculosis* dihydrofolate reductase inhibitor with whole cell activity. PLoS One **7:**e39961.

16. **Vilchèze C, Jacobs WR, Jr.** 2012. The combination of sulfamethoxazole, trimethoprim, and isoniazid or rifampin is bactericidal and prevents the emergence of drug resistance in *Mycobacterium tuberculosis*. Antimicrob Agents Chemother **56:**5142-5148.

17. **Parchina A, Froeyen M, Margamuljana L, Rozenski J, De Jonghe S, Briers Y, Lavigne R, Herdewijn P, Lescrinier E.** 2013. Discovery of an acyclic nucleoside phosphonate that inhibits *Mycobacterium tuberculosis* ThyX based on the binding mode of a 5-alkynyl substrate analogue. ChemMedChem **8:**1373-1383.

18. **McGuigan C, Derudas M, Gonczy B, Hinsinger K, Kandil S, Pertusati F, Serpi M, Snoeck R, Andrei G, Balzarini J, McHugh TD, Maitra A, Akorli E, Evangelopoulos D, Bhakta S.** 2014.

ProTides of N-(3-(5-(2'-deoxyuridine))prop-2-ynyl)octanamide as potential anti-tubercular and anti-viral agents. Bioorg Med Chem **22:**2816-2824.

19. **Alexandrova LA, Chekhov VO, Shmalenyuk ER, Kochetkov SN, El-Asrar RA, Herdewijn P.** 2015. Synthesis and evaluation of C-5 modified 2'-deoxyuridine monophosphates as inhibitors of *M. tuberculosis* thymidylate synthase. Bioorg Med Chem **23:**7131-7137.

20. **Hong W, Wang Y, Chang Z, Yang Y, Pu J, Sun T, Kaur S, Sacchettini JC, Jung H, Lin Wong W, Fah Yap L, Fong Ngeow Y, Paterson IC, Wang H.** 2015. The identification of novel *Mycobacterium tuberculosis* DHFR inhibitors and the investigation of their binding preferences by using molecular modelling. Sci Rep **5:**15328.

21. **Lele AC, Raju A, Khambete MP, Ray MK, Rajan MG, Arkile MA, Jadhav NJ, Sarkar D, Degani MS.** 2015. Design and synthesis of a focused library of diamino triazines as potential DHFR inhibitors. ACS Med Chem Lett **6:**1140-1144.

22. **Mugumbate G, Abrahams KA, Cox JA, Papadatos G, van Westen G, Lelievre J, Calus ST, Loman NJ, Ballell L, Barros D, Overington JP, Besra GS.** 2015. Mycobacterial dihydrofolate reductase inhibitors identified using chemogenomic methods and *in vitro* validation. PLoS One **10:**e0121492.

23. **Raju A, Degani MS, Khambete MP, Ray MK, Rajan MG.** 2015. Antifolate activity of plant polyphenols against *Mycobacterium tuberculosis*. Phytother Res **29:**1646-1651.

24. **Singh V, Brecik M, Mukherjee R, Evans JC, Svetlikova Z, Blasko J, Surade S, Blackburn J, Warner DF, Mikusova K, Mizrahi V.** 2015. The complex mechanism of antimycobacterial action of 5-fluorouracil. Chem Biol **22:**63-75.

25. **Tawari NR, Bag S, Raju A, Lele AC, Bairwa R, Ray MK, Rajan MG, Nawale LU, Sarkar D, Degani MS.** 2015. Rational drug design, synthesis and biological evaluation of dihydrofolate reductase inhibitors as antituberculosis agents. Future Med Chem **7:**979-988.

26. **Machado D, Couto I, Perdigao J, Rodrigues L, Portugal I, Baptista P, Veigas B, Amaral L, Viveiros M.** 2012. Contribution of efflux to the emergence of isoniazid and multidrug resistance in *Mycobacterium tuberculosis*. PLoS One **7:**e34538.